

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
6 January 2005 (06.01.2005)

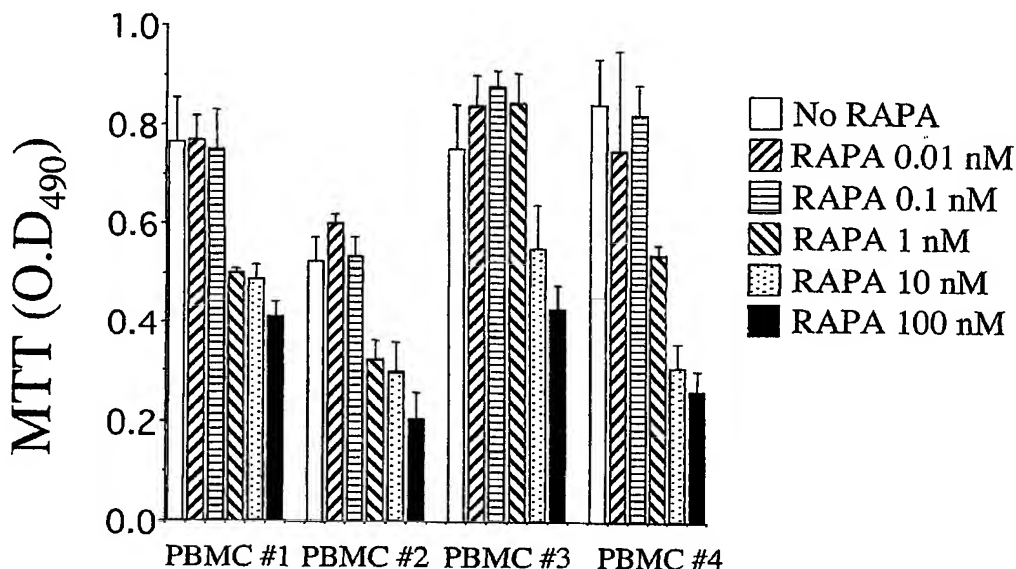
PCT

(10) International Publication Number  
**WO 2005/001027 A2**

- (51) International Patent Classification<sup>7</sup>: C12N MD 20723 (US). HEREDIA, Alonsa [ES/US]; 4955 Linneann Ave NW, Washington, DC 20008 (US).
- (21) International Application Number: PCT/US2004/015681 (74) Agents: FUIERER, Marianne et al.; Intellectual Property Technology Law, P.O. Box 14329, Research Triangle Park, NC 27709 (US).
- (22) International Filing Date: 17 May 2004 (17.05.2004)
- (25) Filing Language: English (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (26) Publication Language: English (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (30) Priority Data: 60/471,453 16 May 2003 (16.05.2003) US
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(54) Title: COMPOSITIONS FOR DOWN-REGULATION OF CCR5 EXPRESSION AND METHODS OF USE THEREFOR



(57) Abstract: The present invention relates to the downregulation of surface receptor CCR5 expression through manipulation of the cell cycle in activated lymphocytes by administering a composition that arrests the G1 phase of the cell cycle, thereby reducing receptor sites for entry of HIV into T cells, and thus, the effects of HIV. Further, a composition is disclosed the includes a G1 phase arresting agent and an antiviral agent, wherein the combination synergically enhances the activity of the antiviral agent.

WO 2005/001027 A2



**Published:**

— without international search report and to be republished  
upon receipt of that report

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

COMPOSITIONS FOR DOWN-REGULATION OF CCR5 EXPRESSION AND  
METHODS OF USE THEREFOR

BACKGROUND OF THE INVENTION

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Field of the Invention

The present invention generally relates to down-regulation of CCR5 expression, and more particularly, to compositions comprising at least one G1 phase arresting agent  
10 that exhibits down-regulation of surface receptor CCR5 expression thereby treating human diseases in which CCR5 receptors plays an adverse role.

Background of the Related Art

15 The human immunodeficiency virus (HIV) has been implicated as the primary cause of the slowly degenerative immune system disease termed acquired immune deficiency syndrome (AIDS). There are at least two distinct types of HIV: HIV-1 and HIV-2. In humans, HIV replication occurs prominently in CD4 T lymphocyte populations, and HIV infection leads to depletion of this cell type and eventually to  
20 immune incompetence, opportunistic infections, neurological dysfunctions, neoplastic growth, and ultimately death.

HIV is a member of the lentivirus family of retroviruses. Retroviruses are small-enveloped viruses that contain a single-stranded RNA genome, and replicate via a  
25 DNA intermediate produced by a virally encoded reverse transcriptase, an RNA-dependent DNA polymerase.

The HIV viral particle comprises a viral core, composed in part of capsid proteins, together with the viral RNA genome and those enzymes required for early replicative  
30 events. Myristylated gag protein forms an outer shell around the viral core, which is, in turn, surrounded by a lipid membrane envelope derived from the infected cell

membrane. The HIV envelope surface glycoproteins are synthesized as a single 160 kilodalton precursor protein, which is cleaved by a cellular protease during viral budding into two glycoproteins, gp41 and gp120. gp41 is a transmembrane glycoprotein and gp120 is an extracellular glycoprotein, which remains non-covalently associated with gp41, possibly in a trimeric or multimeric form.

HIV is targeted to CD4 cells because a CD4 cell surface protein (CD4) acts as the cellular receptor for the HIV-1 virus. Viral entry into cells is dependent upon gp120 binding the cellular CD4 receptor molecules, explaining HIV's tropism for CD4 cells, while gp41 anchors the envelope glycoprotein complex in the viral membrane. CCR5 serves as a co-receptor for the infection of CD4 cells by nonsyncytium-inducing (NSI) strains of HIV-1.

Expression of the CCR5 receptor on T cells is dependent on the activation state of the cells. Resting lymphocytes do not express CCR5, however, upon activation, CCR5 is expressed. The importance of CCR5 for initial transmission of HIV-1 is highlighted by the fact that individuals lacking expression of CCR5 (the CCR5-Δ32 homozygous genotype) are usually resistant to infection (Liu, et al., 1996). In addition, recent studies show that CCR5 cell-surface density correlates with disease progression in infected individuals (Lin, et al., 2002).

Other disorders and the progression of effects have been found to be related to expression of the CCR5 receptor. For example, allograft rejection occurs as a result of extravasation of recipient mononuclear cells into the allograft, a process that is mediated by expression of CCR5 on the infiltrating mononuclear cells. Asthma studies using murine models of allergic airway disease have shown that CCR5 likely plays an important role in airway inflammation. Further, rheumatoid arthritis is characterized by the infiltration of the synovial membrane with mononuclear cells and CCR5 seems to play a role due to the high levels of CCR5 expression found in infiltrated lymphocytes. Interestingly, mononuclear cells present in the active

demyelinating plaques characteristic of subject suffering from multiple sclerosis also show high levels of CCR5 expression.

Thus, it would be advantageous to identify compounds that reduce or inhibit the  
5 expression of CCR5 surface receptors on mononuclear cells and administer such compounds to effect treatment of disorders related to the expression of CCR5 surface receptors.

### SUMMARY OF THE INVENTION

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The present invention relates to the downregulation of surface receptor CCR5 expression through manipulation of the cell cycle in activated lymphocytes by administering a composition that arrests the G1 phase of the cell cycle, thereby disrupting the response of a lymphocyte to IL-2 (through the IL-2R) which governs  
15 the transition from G1 to S phase, as well as the progression through S phase. The reduction of the CCR5 expression reduces receptor sites for entry of HIV into T cells, and thus, the effects of HIV progression.

In one aspect, the present invention relates to suppressing transcription of CCR5, to  
20 reduce expression of CCR5 surface receptors thereby causing an accumulation of chemokines at the cellular level. This accumulation of chemokines is due to reduced number of surface CCR5 receptors for chemokine/ligand uptake.

In another aspect, the present invention relates to suppressing transcription of CCR5,  
25 to reduce expression of CCR5 surface receptors thereby causing a reduced number of surface receptors for binding of HIV gp120, which, in turn, prevents or reduces replication of HIV.

In another aspect, the present invention relates to compositions that inhibit CCR5-  
30 mediated viral entry of HIV by decreasing the number of CCR5 surface receptors

expressed on mononuclear cells including, but not limited to T cells, activated T cells and macrophages.

5 In another aspect, the invention relates to a composition comprising a G1 phase arresting agent that delays entry of the S-phase in a mononuclear cell cycle, wherein the G1 phase arresting agent disrupts signals occurring after binding of IL-2 to the IL-2 receptor (IL-2R) on the cell surface and thus suppresses the expression of CCR5 which is dependent on signaling through the IL-2 receptor.

10 Still another aspect of the present invention relates to a method for inhibiting CCR5-mediated viral entry, namely the downregulation of CCR5 protein expression by the immunomodulatory drug rapamycin (RAPA). RAPA, a bacterial macrolide that is currently approved for the treatment of renal transplantation rejection, exerts cytostatic activity in T cells by disrupting molecular events resulting from the binding of IL-2 to  
15 the IL-2 receptor (Sehgal, S.N., 1998).

The G1 cell cycle modulating agent may include any compound that arrests or prolongs the G1 phase in the cell cycle of mononuclear cells, for example, including but not limited to sodium butyrate, aphidicolin, hydroxyurea (HU), olomoucine,  
20 roscovitine, tocopherols, including alpha-tocopherol, beta-tocopherol, D-alpha-tocopherol, delta-tocopherol, gamma-tocopherol, tocotrienols, rapamycin (RAPA) and functional analogs or derivatives thereof.

The compositions of the present invention may further comprise at least one antiviral  
25 agent. The antiviral agent may include any agent that inhibits entry into a cell or replication therein of an infectious virus, and specifically retroviruses, such as HIV viruses. The antiviral agents include, but are not limited to nucleoside RT inhibitors, CCR5 inhibitors/antagonists, viral entry inhibitors and their functional analogs.

Thus, in one aspect the compositions and methods of the present invention further comprise a therapeutically effective amount of at least one antiviral agent, including, but not limited to: nucleoside RT inhibitors, such as Zidovudine (ZDV, AZT), Lamivudine (3TC), Stavudine (d4T), Didanosine (ddl), Zalcitabine (ddC), Abacavir (ABC), Emirivine (FTC), Tenofovir (TDF), Delaviradine (DLV), Efavirenz (EFV),  
5 Nevirapine (NVP), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir, Combivir (ZDV/3TC), Kaletra (RTV/LPV), Trizivir (ZDV/3TC/ABC);

10 CCR5 inhibitors/antagonists, such as SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857, monoclonal antibodies;

viral entry inhibitors, such as Fuzeon (T-20), NB-2, NB-64, T-649, T-1249, SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857;  
15 and functional analogs or equivalents thereof.

Another aspect of the present invention relates to a method to enhance the efficacy of TAK-779 and decrease the number of CCR5 surface receptors on an activated T cell, the method comprising:

20 administering a composition comprising: a) TAK-779 in an amount found to be ineffective in antagonizing CCR5 receptors and b) a G1 phase arresting agent in an amount effective to reduce expression of CCR5, whereby the inclusion of the G1 phase arresting agent in the composition increases the efficacy of TAK-779. Preferably the G1 phase arresting agent is RAPA or HU and the efficacy is  
25 synergically increased.

In still another aspect, the present invention relates to a method of combating a virus infection wherein CCR5 surface receptor plays an adverse role, comprising:

administering to a patient a composition comprising an effective amount of a  
30 G1 phase arresting compound to reduce expression of CCR5 surface receptors.

In yet another aspect, the present invention relates to a method of maintaining durable viral control of HIV, the method comprising:

administering at least one antiviral agent and a G1 phase arresting compound in a therapeutically effective amount to reduce expression of CCR5 receptors thereby  
5 reducing binding of HIV gp120.

The antiviral agent may be any HIV entry inhibitor, such as TAK 799 or SCH-C both of which block viral binding to CCR5 receptors. Viral resistance to these CCR5 antagonist molecules has been shown to result from more efficient use of CCR5 by  
10 the virus (Trkola, et al., 2002). The fact that HIV-1 viruses are resistant to CCR5 blockers yet still dependant on CCR5 receptors for infection suggests that a decrease in CCR5 will interfere with the growth and emergence of resistant viral variants, thereby increasing the antiviral durability of entry inhibitor therapy.

15 Another aspect of the present invention relates to a therapeutic method to reduce effects and replication of HIV in a HIV infected subject, the method comprising administering a G1 phase arresting agent in combination with the CCR5 antagonist TAK 779 to enhance the efficacy of TAK-779 and a reduction of CCR5 expression.

20 In still a further aspect, the present invention relates to a method of preventing HIV in a subject potentially exposed to the HIV, the method comprising:

administering to the subject at least one G1 phase arresting compound in an effective amount to decrease transcription of CCR5 surface receptors thereby  
inhibiting HIV viral entry into the subject.

25 Other features and advantages of the invention will be apparent from the following detailed description, drawings and claims.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the effect of RAPA on proliferation of PBMCs and reduction of proliferation was noticeable at values greater than 1 nM of RAPA.

5

Figures 2A-D show the effectiveness of RAPA in down-regulating CCR5 expression on T cells and monocytes; 2A shows specific detection of CCR5 surface expression on CD4<sup>+</sup> T cells, from a normal donor, but not on CD4<sup>+</sup> T cells from an individual homozygous for the  $\Delta$ 32 mutation in the CCR5 gene; 2B shows down-regulation of CCR5 surface expression on CD4<sup>+</sup> T cells by RAPA in PBMCs that were cultured for 7 days in the presence of IL-2 and RAPA and then assayed for CCR5 levels, wherein expression of CCR5 on CD4<sup>+</sup> T lymphocytes is shown as a solid line, and fluorescence due to the IgG isotype control is shown as a dashed line; 2C shows inhibition of CCR5 mRNA transcription in PBMCs by RAPA. Total RNA was isolated from PBMCs that had been cultured in the presence of IL-2 and RAPA for 7 days (cells from same experiment as shown in 2B). Equivalent amounts of RNA were subjected to RT-PCR using primer pairs specific for the amplification of CCR5 mRNA (*Upper*) and 18S ribosomal RNA (*Lower*); 2D shows RAPA down-regulates CCR5 cell-surface expression on maturing monocytes that were cultured for 5 days in the presence of RPMI 20/10% ABHS and RAPA were dually immunostained for CD14 and CCR5. Changes in CCR5 surface expression were examined in CD14-gated cells. The immunofluorescence profile obtained with the anti-CCR5 mAb 182 (solid line) is compared with that of the IgG2b isotype control (dashed line). Results in 2B and 2C are representative of data obtained in PBMCs from five different donors. Results in 2D are representative of similar profiles obtained on three different donors.

Figures 3A and 3B shows that RAPA increases extracellular  $\beta$ -chemokine levels in PBMC cultures. 3A shows the results of donor PBMCs that were cultured in the presence of IL-2 and RAPA for 10 days, at which time supernatants were evaluated for  $\beta$ -chemokine content by ELISA and cells were stained for CCR5 expression. Percentage of CD4 lymphocytes expressing CCR5 at each concentration of RAPA is

indicated. Results shown in two donors are representative of four experiments using four different donors. \*,  $P < 0.01$ ; #,  $P < 0.05$ , compared with untreated control by Student's  $t$  test; **3B** shows the effect of RAPA on extracellular levels of MIP-1 $\beta$  in cultures of CCR5-null PBMCs. Levels of MIP-1 $\beta$  protein in the presence and absence  
5 of RAPA were measured in supernatants of IL-2-stimulated PBMCs from a normal donor and from a donor homozygous for the  $\Delta 32$  mutation in the CCR5 gene. Values were obtained on day 10 of culture and are means  $\pm$  SD of duplicate wells.

Figure 4 shows RAPA inhibition of HIV-1 replication in PBMCs, and that the  
10 antiviral activity in R5 HIV-1 is greater than in X4 HIV-1. **4A** shows the results of a replication in seven-day testing period wherein RAPA-treated PBMCs were infected with HIV-1 IIIb or HIV-1 ADA. Infected cells were cultured in the presence of drug RAPA for 7 days, at which time virus replication was measured by p24 and cell viability was measured by the MTT assay. Results (means  $\pm$  SD of triplicate wells)  
15 are representative of seven independent experiments, each on cells from a different donor; **4B** shows effects on HIV-1 IIIb and HIV-1 ADA infected DNase-treated stock of PBMCs when treated with or without 100 nM RAPA. HIV-1 DNA sequences were amplified by PCR in cellular lysates prepared 24 h after infection. Amplified PCR products were detected with a radioactive probe. "+" indicates presence of RAPA in  
20 the PBMC culture before and after infection; "-" indicates no RAPA treatment. Amplification of  $\beta$ -actin sequences indicated same amount of cellular DNA among the different cell lysates (data not shown). NC denotes PCR negative control; **4C** shows the antiviral activity of low concentrations of RAPA when investigated in a panel of R5 strains of HIV-1. Cell proliferation was assayed on uninfected cells from same  
25 donor cultured under identical conditions. Results (means  $\pm$  SD of triplicate wells) are representative of three independent experiments, each on different donor cells.

Figure 5 shows that RAPA inhibits HIV-1 replication in MDMs. Purified monocytes were cultured for 5 days in the presence of RAPA. On day 5, cells were infected with  
30 HIV-1 ADA and cultured in the presence of RAPA for 14 additional days. On days 7, 10, and 14 after infection, virus growth was measured by the RT assay. On day 14,

cell viability was determined by MTT. Results (means  $\pm$  SD) are representative of data obtained in three independent experiments, each using cells from a different donor.

- 5 Figure 6 shows that RAPA enhances the antiviral activity of the CCR5 antagonist TAK-779. PBMCs that had been cultured in the absence or presence of RAPA (1, 10, and 100 nM) for 7 days were infected with HIV-1 ADA in the presence of 0.1 nM TAK-779. Infected cells were cultured in the presence of RAPA and 0.1 nM TAK-779. On day 7 after infection, virus production was measured by the p24 assay in the  
10 culture supernatant. Note the logarithmic scale in the y axis. Data represent means  $\pm$  SD of triplicate wells. Representative results obtained in one of three independent experiments are shown.

- Figure 7 shows that HU enhances the antiviral activity of the CCR5 antagonist TAK-  
15 779. PBMCs that had been cultured in the absence or presence of HU for 7 days were infected with HIV-1 ADA in the presence or absence of TAK-779. On day 7 after infection, virus production was measured by the p24 assay in the culture supernatant.

- Figures 8A - B show the effectiveness of HU in down-regulating CCR5 expression on  
20 T cells and monocytes; 8A shows down-regulation of CCR5 surface expression on CD4<sup>+</sup> T cells by HU in PBMCs that were cultured for 7 days in the presence of IL-2 and HU and then assayed for CCR5 levels, wherein expression of CCR5 on CD4<sup>+</sup> T lymphocytes is shown as a solid line, and fluorescence due to the IgG isotype control is shown as a dashed line; 8B shows inhibition of CCR5 mRNA transcription in  
25 PBMCs by HU. Total RNA was isolated from PBMCs that had been cultured in the presence of IL-2 and HU for 7 days (cells from same experiment as shown in 8A). Equivalent amounts of RNA were subjected to RT-PCR using primer pairs specific for the amplification of CCR5 mRNA (*Upper*) and 18S ribosomal RNA (*Lower*);

- 30 Figure 9 shows changes from baseline of CCR5 mRNA as a measurement of CCR5 expression. Values are normalized for cell house keeping gene beta actin. Fold

differences from baseline ( Day 0) are noted on log scale. Timepoint tested include day 7, 14 and 28 on RAPA and then day 42, 2 weeks post discontinuation of RAPA.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

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A method of treating a viral infection is meant herein to include "prophylactic" treatment or "therapeutic" treatment. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or who exhibits early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

10

The term "therapeutic," as used herein, means a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

15

The term "therapeutically effective amount," as used herein means an amount of compound that is sufficient to provide a beneficial effect to the subject to which the compound is administered. A beneficial effect means rendering a virus incompetent for replication, inhibition of viral replication, inhibition of infection of a further host cell, or increasing CD4 T-cell count, for example.

20

The term "a virally-targeted cell," as used herein, means a cell in which virus is present and is infective or potentially infective and includes epithelial cells, nervous system cells, T-lymphocytes (activated or resting), macrophage, monocytes, tissue dendritic cells or the like.

25

The term "functional equivalent," as used herein, means that the agent retains some or all of the biological activity of the corresponding compound.

30

The term "functional analog," as used herein means compounds derived from a particular parent compound by straightforward substitutions that do not result in a

substantial (i.e. more than 100X) loss in the biological activity of the parent compound, where such substitutions are modifications well-known to those skilled in the art, e.g., esterification, replacement of hydrogen by halogen, replacement of alkoxy by alkyl, replacement of alkyl by alkoxy, etc.

5

The Invention:

#### G1 Phase arresting compounds

- 10 The compositions of the present invention may include any G1 phase arresting agent that arrests, delays or prolongs cell-cycle activity in the G1 phase and/or G1-S interface of mononuclear cells and reduces expression of CCR5. Preferably, G1 phase arresting agent disrupts the response of a lymphocyte to IL-2 (through the IL-2R) which governs the transition from G1 to S phase, as well as the progression through S
- 15 phase.

- G1 phase arresting agents may include, but are not limited to, sodium butyrate, aphidicolin, hydroxyurea (HU), olomoucine, roscovitine, tocopherols, tocotrienols, rapamycin (RAPA) and/or functional analogs thereof. Preferably, the composition
- 20 comprises rapamycin which inhibits the T cell response to IL-2, the substance which triggers T cells already activated by the TCR to progress through G1. Rapamycin therefore stops the cell at the G1-S transition. More preferably, the composition comprises an effective amount of RAPA to disrupt the response of a lymphocyte to IL-2 (through the IL-2R) which governs the transition from G1 to S phase thereby
- 25 causing a reduction of CCR5 expression and concomitantly reducing receptor sites for entry of HIV.

- The present invention employs one of the above-identified G1 phase arresting compound for administration to a subject suffering from a viral infection, wherein the
- 30 compound prolongs the G1 phase of the cell cycle of an activated lymphocyte thereby

inhibiting expression of CCR5 receptors and reducing binding sites for HIV gp120 ligands.

#### Pharmaceutical Compositions

The present invention provides compositions comprising at least one G1 phase arresting compound and optionally at least one antiviral agent, as well as methods of preventing, treating and/or reducing the effects of HIV. The methods comprise administering said compositions comprising the G1 phase arresting compounds and  
10 optionally antiviral agents, wherein the two compounds can be administered, separately, simultaneously, concurrently or sequentially.

#### Pharmaceutically Acceptable Derivatives and Salts

15 The term "pharmaceutically acceptable derivative" is used herein to denote any pharmaceutically or pharmacologically acceptable salt, ester or salt of such ester of a compound according to the invention, or any compound which, upon administration to the recipient, is capable of providing (directly or indirectly) one or more of the compounds according to the invention, or an antivirally active metabolite or residue  
20 thereof.

Preferred esters of the G1 phase arresting compounds of the invention include carboxylic acid esters in which the non-carbonyl moiety of the ester grouping is selected from straight or branched chain alkyl. e.g. n-propyl, t-butyl, n-butyl,  
25 alkoxyalkyl (e.g. methoxymethyl), aralkyl (e.g. benzyl), aryloxyalkyl (e.g. phenoxymethyl), aryl (e.g. phenyl optionally substituted by halogen, C<sub>1-4</sub>alkyl or C<sub>1-4</sub>alkoxy or amino); sulfonate esters such as alkyl- or aralkylsulfonyl (e.g. methanesulfonyl); amino acid esters (e.g. L-valyl or L-isoleucyl); and mono-, di- or triphosphate esters. The phosphate esters may be further esterified by, for example, a  
30 C<sub>1-20</sub> alcohol or reactive derivative thereof, or by a 2,3-di C<sub>2-4</sub> acyl glycerol.

Pharmaceutically acceptable salts include, without limitation, salts of organic carboxylic acids such as acetic, lactic, tartaric, malic, isethionic, lactobionic, p-aminobenzoic and succinic acids; organic sulfonic acids such as methanesulfonic, ethanesulfonic, benzenesulfonic and p-toluenesulfonic acids and inorganic adds such  
5 as hydrochloric, sulfuric, phosphoric and sulfamic acids.

#### Anti-viral compounds

In one aspect, the compositions and methods of the present invention further comprise  
10 a therapeutically effective amount of at least one antiviral agent, including, but not limited to nucleoside RT inhibitors, CCR5 inhibitors/antagonists, viral entry inhibitors and functional analogs thereof.

Preferably, the antiviral agent comprises nucleoside RT inhibitors, such as Zidovudine  
15 (ZDV, AZT), Lamivudine (3TC), Stavudine (d4T), Didanosine (ddI), Zalcitabine (ddC), Abacavir (ABC), Emirivine (FTC), Tenofovir (TDF), Delaviradine (DLV), Efavirenz (EFV), Nevirapine (NVP), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir, Combivir (ZDV/3TC), Kaletra (RTV/LPV), Trizivir (ZDV/3TC/ABC);

20 CCR5 inhibitors/antagonists, such as SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857, monoclonal antibodies;

viral entry inhibitors, such as Fuzeon (T-20), NB-2, NB-64, T-649, T-1249, SCH-C,  
25 SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857; and functional analogs thereof.

#### Antiviral Therapy

30 Although current treatment with antiretroviral (ARV) therapy causes suppression of HIV replication and results in improvements of immune function, it is limited by high

costs, toxicities and adherence difficulties. Moreover, the chance of achieving long-term control of HIV infection with antiretroviral therapy alone seems very unlikely. To date, current antiretroviral therapy has been shown to be insufficient to completely eradicate HIV from infected individuals and there is no real data that the amount of residual virus is decreasing with time on typical antiretroviral therapy. Further, after  
5 stopping antiretroviral therapy, the viral load can rebound to higher levels than pretreatment viral loads (Davey, 1999; Dybul, et al., 2002 and 2001).

Antiretroviral therapy demands stringent adherence to complex dosing regimens. The  
10 rate of virological failure over a 6-month period of time has been demonstrated to be as high as 60% in patients that cannot achieve greater than 95% adherence. The combination of multiple adverse side effects associated with antiretroviral therapy and the availability of this treatment to only 1 in 20 of the estimated 33 million people infected world wide has prompted reconsideration of the current strategies for  
15 achieving the goals of HIV therapy.

Moreover, HIV therapy is now thought to be a life-long process. Therefore, it is crucial to develop effective treatments that can be successfully administered for long periods of time for the suppression of retroviruses, and in particular, the prevention  
20 and/or inhibition of HIV. Further, it is desirable to eliminate, or at least minimize, the cytotoxicity associated with the administration of antiviral agents otherwise determined to be effective. It is generally recognized that the toxicity of an antiviral agent may be avoided or at least minimized by administration of a reduced dose of the antiviral agent; however, it is also recognized that the effectiveness of an antiviral  
25 agent generally decreases as the dose is reduced.

Thus, one embodiment of the present invention provides for reducing the dose of antiviral agents while maintaining or reducing viral load by using cyclic therapy and introducing the G1 cell cycle agents of the present invention to a dosing regime for an  
30 HIV infected subject. Specifically, the use of the G1 phase arresting compounds in combination with antiviral agents has shown promise to maintain viral suppression in

a cycle therapy dosing program. By using 50% less medication, side effects associated with antiretroviral use have been shown to be reduced and adherence has shown to be increased. The other obvious impact is on overall cost of medications, which will facilitate expanding these drugs throughout the developed world.

5

Thus, in one embodiment of the present invention, cyclic therapy is employed as an alternative approach designed to increase activity of antiviral agents, decrease drug cost and toxicity. Furthermore, since one component of the compositions of the present invention targets cellular machinery of the host, rather than the virus, the present inventors expect that viral resistance to this drug combination essentially  
10 would not occur.

A cycle antiviral therapy regime can run for about 12 weeks and then a G1 phase arresting compound is added or substituted for four weeks. If the viral load remains  
15 low or approximately constant, the cycles can be altered to reduce the time period of each cycle. The time period for consumption of the antiviral drugs can be reduced, if augmented with a G1 phase arresting compound. Furthermore, a time period can be introduced that includes no antiviral drugs and only a G1 phase arresting compound. This time period wherein no antiviral agents are consumed by the subject, provides  
20 the biological system of the subject sufficient time to repair or compensate for the toxic effects of the antiviral compound.

Further, the compositions and methods of the present invention can be used to treat HIV viral infections by reducing viral load and replication of the virus by reducing  
25 binding sites for gp120 ligands.

Doses to be administered are variable according to the G1 phase arresting agent, the antiviral agent, the treatment period, frequency of administration, the host, and the nature and severity of the infection. The dose can be determined by one of skilled in  
30 the art without an undue amount of experimentation.

The compositions of the invention are administered in substantially non-toxic dosage concentrations sufficient to ensure the release of a sufficient dosage unit of the present combination into the patient to provide the desired inhibition of the HIV virus. The actual dosage administered will be determined by physical and physiological factors such as age, body weight, severity of condition, and/or clinical history of the patient. The active antiviral components are ideally administered to achieve *in vivo* plasma concentrations of an antiviral agent of about 0.01  $\mu$ M to about 100  $\mu$ M, more preferably about 0.1 to 10  $\mu$ M, and most preferably about 1-5  $\mu$ M, and of a G1 phase arresting agent of about 1  $\mu$ M-25 $\mu$ M, more preferably about 2-20  $\mu$ M, and most preferably about 5-10  $\mu$ M.

For example, in the treatment of HIV-positive and AIDS patients, the methods of the present invention may use compositions to provide from about 0.005-500 mg/kg body weight/day of an antiviral agent, more preferably from about 0.1-200 mg/kg/day, and most preferably 1-50 mg/kg/day; and from about 0.01-1000 mg/kg body weight/day of a G1 phase arresting agent, more preferably from about 0.001-1000 mg/kg/day, or most preferably from about 0.5-50 mg/kg/day. Particular unit dosages of a G1 phase arresting agent and an antiviral agent of the present invention include 50 mg, 100 mg, 200 mg, 500 mg, and 1000 mg amounts, for example, formulated separately, or together as discussed *infra*.

It will be understood, however, that dosage levels that deviate from the ranges provided may also be suitable in the treatment of a given viral infection.

Therapeutic efficacy of the G1 phase arresting compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining The LD50 (The Dose Lethal To 50% Of The Population) and The ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds, which exhibit large therapeutic indexes, are preferred. The data obtained from the cell culture assays and animal studies can be

used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any  
5 compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more  
10 accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The desired dose is preferably presented as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may  
15 be administered in unit dosage forms, for example, containing 0.01 to 1000 mg, preferably 1 mg to 50 mg, depending on the number of sub-doses, of the G1 phase arresting compound per unit dosage form.

While it is possible for the specific G1 phase arresting compound and antiviral agent  
20 to be administered individually, either sequentially or simultaneously, it is preferable to present them together, as combined in a pharmaceutical composition.

The compositions of the present invention may comprise both the above-discussed ingredients, together with one or more acceptable carriers thereof and optionally other  
25 therapeutic agents. Each carrier must be "pharmaceutically acceptable " in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject.

The present invention provides a method for the treatment or prophylaxis of a viral  
30 infection such as retroviral infections which may be treated or prevented in accordance with the invention include human retroviral infections such as human

immunodeficiency virus. The specific G1 phase arresting compounds, compositions and methods according to the invention are especially useful for the treatment of AIDS and related HIV-positive conditions. The compounds of the present invention are also useful for the treatment of asymptomatic infections or diseases in humans  
5 caused by or associated with human retroviruses.

The therapeutic compositions according to the present invention may be employed in combination with other-therapeutic agents for the treatment of viral infections or conditions. Examples of such further therapeutic agents include agents that are  
10 effective for the treatment of viral infections or associated conditions such as immunomodulatory agents such as thymosin, ribonucleotide reductase inhibitors such as 2-acetylpyridine 5-[(2-chloroanilino) thiocarbonyl) thiocarbonohydrazone, interferons such as alpha -interferon, 1- beta -D-arabinofuranosyl-5-(1-propynyl)uracil, 3'-azido-3'-deoxythymidine, ribavirin and phosphonoformic acid.

15

#### Routes of Administration

The compositions according to the present invention, may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including transdermal,  
20 buccal and sublingual), vaginal and parenteral (including subcutaneous, intramuscular, intravenous and intradermal). It will be appreciated that the preferred route will vary with the condition and age of the recipient, the nature of the infection and the chosen active ingredient.

25 Pharmaceutical formulations of the present invention include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration.

30 The formulations may conveniently be presented in unit dosage form and may be prepared by methods known in the art of pharmacy. Such methods include the step of

bringing into association the G1 phase arresting compound and optionally an antiviral agent with the carrier. The carrier optionally comprises one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the separate ingredients with liquid carriers or finely divided  
5 solid carriers or both, and then if necessary shaping the product.

Compositions suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches suitably contain the G1 phase arresting  
10 compound and optionally an antiviral agent such as a CCR5 antagonist: 1) in an optionally buffered, aqueous solution; or 2) dissolved and/or dispersed in an adhesive; or 3) dispersed in a polymer.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, caches or tablets, each containing a  
15 predetermined amount of the ingredients; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion.

20 A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the G1 phase arresting compound and antiviral agent in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent,  
25 preservatives, disintegrant (e.g. sodium starch glycollate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or  
30 controlled release of one or more of the ingredients therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release

profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

5 Formulations suitable for topical administration in the mouth include lozenges comprising one or more of the G1 phase arresting compounds and optionally an antiviral agent in a flavored basis, usually sucrose or acacia; pastilles comprising one or more of the ingredients in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the one or more of the ingredients in a suitable liquid carrier.

10

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

15 Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing, in addition to the one or more of the compounds of the present invention, such carriers as are known in the art to be appropriate.

20 Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multidose sealed containers, for example, ampules and vials, and may  
25 be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

30 For a perinatal subject, the drug combination of the present invention may be, for example, administered orally after 36 weeks of pregnancy and continued through

delivery. Interventions around the time of late gestation and delivery (when the majority of transmissions are thought to occur) are most efficacious.

In addition to the compositions described previously, the compounds may also be  
5 formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for  
10 example, as a sparingly soluble salt. Other suitable delivery systems include microspheres that offer the possibility of local noninvasive delivery of drugs over an extended period of time. The administered therapeutic is slowly released from these microspheres and taken up by surrounding tissue cells (e.g. endothelial cells).

15 The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

20 Suitable G1 cell cycle agents, can be used in HIV treatment strategies that allow for continued viral suppression to be maintained with less dependence on combination antiretroviral (ARV) therapy. The current goal of ARV is to obtain viral suppression as low as possible for as long as possible. Requiring less frequent dosing or a decreased quantity of ARV to control viral suppression directly addresses the  
25 problems, set forth below, associated with achieving the current goals of antiretroviral therapy including:

1. Current regimens of HAART are cumbersome and complicated and require sustained tolerance and strict adherence to 3 or more drugs.
2. Long term tight adherence may be impossible for most patients.
- 30 3. Long term tolerance to accumulating medication toxicities may be impossible for most patients.

4. Current treatment guidelines for HIV infection recommend a relatively late initiation of HAART because of the inability to eradicate the infection with HAART alone and the risk of drug-related side-effects, including serious metabolic syndromes.
5. Some patients who have not been treated until later stages of the disease will have a high level of viral load, which could increase the risk of transmission and cause a public health problem.
6. Lastly, the vast majority of HIV infected people worldwide have no access to antiretroviral drugs due mostly to cost.

10

By incorporating G1 cell phase arresting agents into therapeutic approaches with the focus shifted towards maintaining long term viral control, with less complex, less toxic, and more affordable regimens, that can be applicable throughout the world. The present invention that targets the G1 cellular cycle to reduce expression of CCR5

15 receptors in activated T Cells can be used successfully to maintain viral suppression in chronic HIV-1 infection without the need of continuous therapy with multiple antiretroviral drugs. These results have a positive impact on cost, side effects, and availability of HIV therapy.

- 20 The present invention is further illustrated by the following examples that should not be construed as limiting in any way.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic

25 biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984);

30 Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins

eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

## EXAMPLES

### Methods and Materials

15

Cell Culture and Flow Cytometry: Cultures of peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages (MDMs) were performed on normal donors as described (Poli, 1993; Perno, 1993). PBMCs were maintained in the presence of 100 units/ml rhIL-2 (Roche Molecular Biochemicals). Cell viability was determined by Trypan blue staining or by the MTT assay (Roche Molecular Biochemicals).

20

RAPA was purchased from Calbiochem. The CCR5 antagonist, TAK-779 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD).

25

CCR5 surface expression was measured on PBMCs cultured in the presence of IL-2 for 7-10 days. Staining was done as described (Lane, 1999) but using CCR5 mAb 182 (R & D Systems). Background staining was determined by adding an isotype-matched control (IgG $\gamma$ h, R&D Systems) instead of the anti-CCR5 mAb. Data were acquired by

using a FACSCalibur flow cytometer (BD Biosciences) and analyzed by using FLOWJO (Tree Star, San Carlos, CA).

Levels of the  $\beta$ -chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES were measured in culture supernatants by using ELISA kits (R & D Systems).

- 5    Infectivity Assays: The following viruses were used in infection experiments: HIV-1 IIIb, HIV-1 ADA, HIV-1 BaL, HIV-1 JRFL, HIV-1 JRCSF, and HIV-1 SF162. HIV-1 IIIb is a T cell line-adapted lab strain that uses CXCR4 for entry into cells, whereas the rest are isolates that use CCR5. Viruses were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.
- 10   For infection of PBMCs, fresh donor PBMCs were cultured for 7 days in medium containing IL-2. On day 7, cells were exposed to the virus for 3 h. Nonadsorbed virus was removed by washing cells with PBS three times. Infected cells were cultured in IL-2 medium. Infection of MDMs was carried out as described before (Perno, 1993). Unless otherwise indicated, PBMCs were infected by using an moi of 0.001, and
- 15   MDM were infected by using an moi of 0.002. Virus growth was monitored in culture supernatants by measuring p24 antigen levels by ELISA (NEN) or by measuring viral RT activity in an RT assay (Willey, 1988).

- PCR Methodologies: Amplification of CCR5 and  $\beta$ -chemokine RNA sequences was performed by RT-PCR as described (Baba, 1996; Levine, 1996). In some
- 20   experiments, the effect of RAPA treatment on virus entry in PBMCs was investigated by DNA PCR. Briefly, PBMCs that had been treated with IL-2 and RAPA for 7 days were infected for 3 h with HIV-1 IIIb or HIV-1 ADA at an moi of 0.05. Virus inocula had been first filtered through a 0.22- $\mu$ m filter and then treated with DNase (10  $\mu$ g/ml) for 30 min at 37°C to decontaminate the inoculum of HIV-1 DNA. Infected cells were
- 25   washed extensively to remove residual virus. At 24 h after infection, cell lysates were prepared, and aliquots were amplified by DNA PCR using primer pair M661/M667 (Zack, 1990). Amplified products were detected by liquid hybridization using a <sup>32</sup>P-labeled probe (Spina, 1995). Intensities of hybridization signals were measured in a

phosphoimager.  $\beta$ -Actin primers were used to control for DNA amount input in the sample.

### Example 1

Effect of RAPA on PBMC Proliferation and Viability: Purified PBMCs from normal donors were cultured in the presence of IL-2 and RAPA (10-fold serial dilutions, from  $10^4$  to 0.01 nM). On day 7, the extent of cell proliferation was measured by the MTT assay. Representative results obtained on one of two independent experiments, each using cells from four donors, are shown. For each donor, data values are mean  $\pm$  SD of three independent wells. Reduced proliferation, measured by the MTT assay on day 7, was detected at drug concentrations  $\geq 1$  nM as shown in Figure 1. Drug toxicity was observed at drug concentrations above  $10^3$  nM (data not shown).

### Example 2

RAPA Down-Regulates CCR5 Expression on T Lymphocytes and Monocytes: The specificity of the CCR5 was determined using surface-staining protocol by measuring CCR5 expression on lymphocytes from a normal donor and from a donor previously characterized as homozygous for the  $\Delta 32$  mutation in the CCR5 gene. Before staining, PBMCs from both donors were cultured for 7 days in the presence of IL-2 because these culture conditions up-regulate CCR5 surface expression (Bleul, 1997). Results, depicting CCR5 expression on gated CD4 T cells, are shown in Figure 2A. The results show specific detection of CCR5 surface expression on CD4<sup>+</sup> T cells from a normal donor, but not on CD4<sup>+</sup> T cells from an individual homozygous for the  $\Delta 32$  mutation in the CCR5 gene. The results indicate that the methodology used in the present example can specifically detect CCR5 surface expression.

To determine the effect of RAPA on CCR5 surface expression on lymphocytes from normal donors, fresh donor PBMCs were cultured in IL-2 medium in the presence of increasing concentrations of RAPA (0.1, 1, 10, and 100 nM) for 10 days. On days 7 and 10, CCR5 surface expression on CD4 and CD8 T lymphocytes was measured by dual staining with anti-CD4 and anti-CD8 antibodies in combination with anti-CCR5

mAb 182 and analysis on the FACS. Day 7 and 10 results indicated that RAPA concentrations  $\geq 1$  nM down-regulated CCR5 surface expression on CD4 lymphocytes in the five donors tested. RAPA at 0.1 nM down-regulated CCR5 protein expression on CD4 lymphocytes from some but not all donors. Representative day 7 results, showing concentrations of RAPA that effectively down-regulated CCR5 in all donors, are depicted in Figure 2B. A similar decrease on CCR5 expression was evident on the CD8 lymphocyte subset, and CCR5 down-regulation in both CD4 and CD8 lymphocyte subsets was also observed on day 10 (data not shown).

At the transcription level, semiquantitative RT-PCR analysis of RNA isolated from RAPA-treated PBMC cultures showed decreased amounts of CCR5 transcripts in the presence of drug (Figure 2C *Upper*). RT-PCR analysis of ribosomal 18S RNA indicated similar RNA content among samples yielding reduced levels of CCR5 transcripts (Figure 2C *Lower*). In addition, amplification of RNA samples in the absence of the RT step gave no amplification signal, thus ruling out the possibility of cellular DNA contamination in the RNA preparations (data not shown).

Similarly, RAPA down-regulates CCR5 cell-surface expression on maturing monocytes. Monocytes cultured for 5 days in the presence of RPMI 20/10% ABHS and RAPA were dually immunostained for CD14 and CCR5. Changes in CCR5 surface expression were examined in CD14-gated cells. The immunofluorescence profile obtained with the anti-CCR5 mAb 182 (solid line) is compared with that of the IgG2b isotype control (dashed line), as shown in Figure 2D. Monocytes cultured for 5 days in the presence of RAPA showed reduced levels of CCR5 surface expression as compared with the drug-untreated cultures. Experiments using monocytes from three different donors showed consistent down-regulation of CCR5 surface expression at RAPA concentrations as low as 0.01 nM. These results show that RAPA reduces CCR5 surface expression on cultured T lymphocytes (both CD4 and CD8) and monocytes. Together with the RT-PCR results in PBMCs, these results indicate that RAPA interferes with CCR5 expression by reducing gene transcription.

### Example 3

RAPA Increases Extracellular Levels of MIP-1 $\alpha$  and MIP-1 $\beta$  in PBMC Cultures:

Because lymphocytes and monocytes cultured in the presence of RAPA presented reduced CCR5 RNA and protein levels, the levels of the CCR5 ligands MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES were measured in supernatants of RAPA-treated PBMC cultures.

5 PBMCs from four donors were cultured in the presence of IL-2 and RAPA for 10 days. On day 10, percentage of cells expressing CCR5 and supernatant chemokine content were determined for each donor. As in previous experiments, RAPA treatment resulted in reduced levels of CCR5 protein expression. When chemokine content in culture supernatants was measured, it was found that MIP-1 $\alpha$  and MIP-1 $\beta$   
10 levels were higher in the presence of RAPA than in its absence in all four donors. Among the different donors, RAPA-treated cultures contained 6-39-fold higher levels of MIP-1 $\alpha$  than untreated cultures. Similarly, MIP-1 $\beta$  levels were increased 17-47-fold in the presence of RAPA as compared with untreated controls. In contrast, levels of RANTES in the presence of RAPA were increased in two donors while remaining  
15 unchanged or even decreasing in the others. Chemokine results obtained in two of the donors, showing disparity of RANTES levels in the presence of RAPA, are depicted in Figure 3A.

To determine whether the increased levels of MIP-1 $\alpha$  and MIP-1 $\beta$  proteins detected in the supernatants of RAPA-treated cells were the result of increased transcriptional  
20 activity, total RNA from RAPA-treated and untreated cultures was amplified by semiquantitative RT-PCR. Amplification of RNA isolated in days 3, 7, and 10 of culture with primer pairs specific for MIP-1 $\alpha$  and MIP-1 $\beta$  showed no differences in transcript amount for either chemokine between RAPA-treated and untreated cultures (data not shown). These data suggested that RAPA does not augment extracellular  
25 MIP-1 $\alpha/\beta$  levels by enhancing the production of chemokine transcripts.

The above results, showing that RAPA treatment of cells resulted in reduced amounts of CCR5 transcripts (and protein levels) without enhancing the production of chemokine transcripts, suggested that the observed increases on MIP-1 $\alpha/\beta$  proteins is due to a lack of chemokine uptake on RAPA-treated cells. The above results were  
30 confirmed by evaluating the effect of RAPA on the secretion of MIP-1 $\beta$ , a chemokine

that uses CCR5 as its only receptor, in cultures of PBMCs derived from a donor homozygous for the  $\Delta 32$  mutation in the CCR5 gene. In this experimental setting, in which MIP-1 $\beta$  cannot be endocytosed by CCR5, the effect of RAPA on MIP-1 $\beta$  protein levels provided information regarding the mechanism by which RAPA increases  $\beta$ -chemokine levels. To this end, stimulated PBMCs from two normal donors and from a CCR5-null donor were cultured in the presence of RAPA as in previous experiments. MIP-1 $\beta$  levels in supernatants were evaluated on day 10. Representative results obtained in one of the normal donors are shown in Figure 3B next to the results obtained on the CCR5-null donor. In the normal donor, RAPA treatment resulted in an increased level of MIP-1 $\beta$  protein (9.3-fold increase as compared with the RAPA-untreated control) as expected from previous experiments. However, MIP-1 $\beta$  levels in the CCR5-null donor were only increased by 1.2-fold in the presence of RAPA. Together, these results show that increased levels of MIP-1 $\beta$  protein, and additionally MIP-1 $\alpha$ , in the presence of RAPA reflect chemokine accumulation due to diminished uptake by cells presenting reduced levels of CCR5 co-receptor.

#### Example 4

Antiviral Activity of RAPA in PBMCs: The antiviral activity of RAPA was assayed in PBMCs that had been cultured in the presence of RAPA for 7 days before infection. Cells were infected with the X4 HIV-1 IIIb and the R5 HIV-1 ADA strains. Infected cells were cultured in the presence of RAPA (same concentration as during pretreatment) for 7 additional days, during which time virus replication and cell viability were measured. In a total of seven different experiments using cells from different donors, the antiviral effect of RAPA was more potent against HIV-1 ADA than against HIV-1 IIIb. The results shown in Figure 4A for one donor, show that at 10 nM RAPA, the average value of HIV-1 ADA inhibition in the seven experiments was 91% (range of 88-97%), whereas at 100 nM RAPA, HIV-1 ADA was inhibited by 94% (range of 92-99%). In contrast, 10 nM RAPA inhibited HIV-1 IIIb by 13.5% (range of 5-25%), and 100 nM RAPA inhibited HIV-1 IIIb by 32% (range 29-60%).

To further demonstrate the disproportionate antiviral effect of RAPA on R5 versus X4 HIV-1 strains, the antiviral effect of the drug was next assessed by measuring viral DNA in cells shortly after infection. Donor PBMCs that had been cultured for 1 week in the presence (100 nM RAPA) or absence of drug were infected with DNase-treated stocks of R5 HIV-1 ADA or X4 HIV-1 IIIb. At 24 h after infection, cell lysates were prepared and amplified for HIV-1 DNA sequences by PCR. Amplified PCR products were detected by using a radiolabeled probe. As shown in Figure 4B, phosphorimager analyses of the radioactive signals indicated that HIV-1 IIIb DNA content was the same in the RAPA-treated and untreated cells. In contrast, HIV-1 ADA DNA content in the RAPA-treated cells was three times lower than in the untreated cells. Primer pairs specific for the  $\beta$ -actin gene indicated the same DNA input among samples (data not shown).

As the results obtained with HIV-1 IIIb and HIV-1 ADA suggested that RAPA exerted a more potent antiviral effect in R5 than in X4 HIV-1, the antiviral activities of low concentrations of RAPA (0.01, 0.1, and 1 nM) were next evaluated against a panel of five R5 strains of HIV-1, with the results shown in Figure 4C. At these concentrations of RAPA, antiviral activity was seen against R5 strains but not against HIV-1 IIIb. RAPA at 0.01 nM inhibited R5 HIV-1 by 10-64% depending on the strains, whereas 0.1 nM RAPA inhibited virus replication by 15-85%. At 1 nM RAPA, all R5 viruses were inhibited by  $\geq 90\%$ . Together, these results demonstrate that RAPA decreases the susceptibility of PBMCs to be infected by CCR5-using strains of HIV-1 while having little effect in CXCR4-using strains.

#### Example 5

Antiviral Activity of RAPA in Macrophages: The antiviral activity of RAPA in MDMs was first assayed under the culture conditions shown to down-regulate expression of CCR5 (see above results). To this end, donor monocytes were cultured for 5 days in the presence of RAPA. On day 5, cells were infected with HIV-1 ADA. Infected cells were cultured in the presence of RAPA for an additional 14 days. Virus production was measured on the culture supernatants on days 7, 10, and 14 after

infection. Cell viability was measured by the MTT assay at the end of the experiment. Over the course of the experiment, RAPA inhibited virus replication in a dose-dependent manner. On day 14, RAPA concentrations ranging 0.1-100 nM inhibited virus production by 70-95%, as shown in Figure 5. Cell viability at the end of  
5 experiment was reduced at RAPA concentrations  $\geq 10$  nM. In an additional experiment in which RAPA was used at 0.01 nM, the R5 viruses HIV-1 ADA and HIV-1 SF 162 were inhibited by 64% and 45%, respectively (data not shown).

In the above-described experiment, monocytes had been pretreated with RAPA during the 5-day differentiation period. To control for the possible interference of RAPA  
10 with the process of monocyte differentiation, a new infection experiment in which RAPA was not present during the 5-day monocyte differentiation period was designed. To this end, fresh monocytes were cultured for 5 days in the absence of RAPA. On day 5, cells were infected with HIV-1 ADA and then exposed to RAPA. Under these experimental conditions, two independent experiments using monocytes from two  
15 different donors indicated that 1 nM RAPA inhibited virus replication by ~60% in one of the donors and by ~80% in the other donors (virus production measured on day 14 after infection; data not shown).

Taken together, these results show that RAPA treatment of differentiating monocytes interferes with their ability to become susceptible targets for HIV infection and that  
20 RAPA also interferes with the ability of HIV to replicate in already differentiated macrophages.

### Example 6

RAPA Enhances the Antiviral Activity of the CCR5 Antagonist TAK-779: Testing was conducted to determine whether the down-regulation of CCR5 surface expression  
25 observed in the presence of RAPA would increase the potency of a CCR5 antagonist drug. To test this hypothesis, donor PBMCs were cultured in IL-2 medium in the absence or presence of RAPA (1, 10, and 100 nM). After 7 days, cells were infected with HIV-1 ADA in the presence of 0.1 nM TAK-779, a concentration of the drug TAK-779 showing little antiviral activity. Infected cells were cultured in the presence

of RAPA (same concentration as during pretreatment) plus 0.1 nM TAK-779. Virus production was determined 7 days after infection. The results as set forth in Figure 6 show that in the absence of RAPA, 0.1 nM TAK-779 caused a 21% inhibition of virus replication. However, in the presence of 1 nM RAPA, the antiviral effect due to TAK-  
5 779 increased from 21% to 74.5% virus inhibition. Similarly, the antiviral activity of TAK-779 was increased to 89% and 96% virus inhibition in the presence of 10 and 100 nM RAPA, respectively. The TAK-779 concentration used did not affect cell viability (data not shown). These results suggest that the antiviral properties of a CCR5 antagonist drug are enhanced by RAPA.

10

The results shown in Figure 6 illustrate synergic efficacy with the combination of RAPA and TAK 779. As stated above, 0.1 nM TAK-779 shows little antiviral activity and the results shown in Figure 4 indicate that administering RAPA alone reduces the level of P24 to nanograms/ml amounts. However, the combination of  
15 both compounds reduces the levels of p24 to picograms/ml. Thus, the combination provides for a synergic reduction in replication of HIV-1.

### Example 7

20 The synergic activity of RAPA and TAK-779 in reducing replication of HIV-1 is further shown with the combination of HU and TAK-779. Figure 7 illustrates that HU synergically enhances the antiviral activity of the CCR5 antagonist TAK-779. Donor PBMCs were cultured in IL-2 medium in the absence or presence of HU ( 0 and 10  $\mu$ M). After 7 days, cells were infected with HIV-1 ADA. Infected cells were cultured  
25 in the presence of HU (same concentration as during pretreatment) plus different concentration of TAK-779 ranging from 0, 2 and 20 nM). Virus production was determined 7 days after infection. Combinations of TAK-779 and HU showed that without HU and TAK-799 the p24 value was approximately 5000 picograms/ml. The introduction of 10  $\mu$ M of HU reduced the levels of p24 approximately 60%. The  
30 introduction of 2 nM of TAK-779 reduced the levels of p24 about 20%. However, the combination of 10  $\mu$ M of HU and 2 nM of TAK-779 caused a overall reduction of

approximately 90 % of p24, thus indicating that the combination caused a synergic reduction in p24 levels and increased the activity of the CCR5 antagonist TAK-779. Thus, these results show a synergic increase in the activity of CCR5 antagonist.

## 5 Example 8

To determine the effect of HU on CCR5 surface expression on lymphocytes from normal donors, fresh donor PBMCs were cultured in IL-2 medium in the presence of increasing concentrations of HU (0, 50, 100 and 200  $\mu$ M) for 10 days. On days 7 and 10, CCR5 surface expression on CD4 and CD8 T lymphocytes was measured by dual staining with anti-CD4 and anti-CD8 antibodies in combination with anti-CCR5 mAb 182 and analysis on the FACS. Day 7 and 10 results indicated that HU concentrations  $\geq 50$   $\mu$ M down-regulated CCR5 surface expression on CD4 lymphocytes in the five donors tested. At the transcription level, semiquantitative RT-PCR analysis of RNA isolated from HU-treated PBMC cultures showed decreased amounts of CCR5 transcripts in the presence of drug (Figure 8B *Upper*). RT-PCR analysis of ribosomal 18S RNA indicated similar RNA content among samples yielding reduced levels of CCR5 transcripts (Figure 8B *Lower*).

## 20 Example 9

*In vivo* effects of Rapamycin on expression of the Chemokine receptor 5 (CCR5) and accumulation of chemokines MIP 1 $\alpha$ , MIP-1 $\beta$  and RANTES due to diminished uptake by cells presenting reduced expression levels of CCR5 co-receptor in volunteers with chronic HIV infection HIV-1 has been shown in most instances to use the chemokine receptor, CCR5, as a co-receptor for entry into macrophages and CD4 lymphocytes. The natural ligands for the CCR5 co-receptor are proteins called  $\beta$ -Chemokines. In the *in vitro* models, discussed above, it was demonstrated that Rapamycin, as well as other G1 cell cycle agents including hydroxyurea markedly decreased expression of CCR5 surface receptors as shown in Figures 2 B-C and 8 A-B.

To assess the expression activity of CCR5 and its effects on levels of CCR5 receptors *in vivo*, an open-labeled, non-randomized proof of concept trial was performed in which 8 volunteers with established HIV infection were given 2 mg/day of Rapamycin, following a 6 mg loading dose, for 28 days. Peripheral blood for determining expression levels of CCR5 was obtained at the screening visit, days 7, 14 and 28 of the Rapamycin dosing, and at day 42 (which was two weeks following the last dose of Rapamycin). Eight subjects were enrolled in the study and to date 3 volunteers have completed the study and Rapamycin was well-tolerated.

10

Figure 9 depicts alteration in CCR5 expression observed in HIV infected volunteers during this trial. All three volunteers demonstrated a decrease in CCR5 expression by the latest day 28 which was associated with use of RAPA. Clearly, volunteer 001 and 006 experienced an almost immediate reduction in CCR5 expression, while volunteer 007 experienced a reduction later in the treatment. Thus, altering the cell-cycle of peripheral blood mononuclear cells with a G1-specific agent, Rapamycin, resulted in the decreased RNA expression of CCR5 in HIV infected volunteers with established HIV infection; and, this agent was well-tolerated.

20 The targeting by RAPA of a cellular component such as CCR5, as opposed to targeting of the virus itself, offers an antiviral strategy that is less likely to lead to virus resistance, as cellular components are not expected to mutate under drug pressure. The *in vitro* studies suggest that RAPA would be more effective in controlling the replication of R5 than X4 strains of HIV-1. In this regard, the therapeutic use of RAPA as a treatment of early HIV-1 disease (before appearance of X4 strains) is of great value, particularly in light of current guidelines that advocate delayed initiation of antiretroviral therapy (Dybul, 2002). Furthermore, the antiviral properties of RAPA are especially relevant in geographical areas where subtype C HIV-1 is present, as these viruses use CCR5 as major co-receptor (Bjornal, 1999). Subtype C HIV-1

infections have risen in prevalence over the last decade, and they currently constitute the predominant subtype worldwide (Essex, M. (1999).

Moreover, the antiviral properties of RAPA provide new treatment opportunities for suppression of allograft rejection in HIV-infected subjects undergoing solid organ  
5 transplantation. The antiviral properties of RAPA, coupled with its antiangiogenic properties (Guba, 2002), suggest that RAPA offers a better choice for HIV patients undergoing organ transplantation.

In summary, the ability of RAPA to down-regulate CCR5 co-receptor expression and to augment extracellular levels of  $\beta$ -chemokines offers a new strategy with important  
10 implications for the treatment and prevention of HIV-1 infection. The combination of RAPA and CCR5 antagonists is especially effective in controlling virus replication in patients.

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All references cited herein are hereby incorporated by reference herein for all purposes.

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## CLAIMS OF THE INVENTION

That which is claimed is:

- 5 1. A pharmaceutical composition for decreasing expression of CCR5 surface receptors on mononuclear cells, the composition comprising a therapeutically effective amount of at least one G1 phase arresting compound.
2. The pharmaceutical composition of claim 1, further comprising at least one  
10 antiviral agent.
3. The pharmaceutical composition of claim 1, wherein the G1 phase arresting compound is a member selected from the group consisting of sodium butyrate, aphidicolin, hydroxyurea (HU), olomoucine, roscovitine, tocopherols, tocotrienols,  
15 and rapamycin (RAPA).
4. The pharmaceutical composition of claim 2, wherein the antiviral agent is an HIV antiviral agent.
- 20 5. The pharmaceutical composition of claim 4, wherein the HIV antiviral agent is a nucleoside RT inhibitor, CCR5 inhibitors/antagonist, viral entry inhibitor or functional equivalent thereof.
6. The pharmaceutical composition of claim 2, wherein the antiviral agent is at  
25 least one member selected from the group consisting of: Zidovudine (ZDV, AZT), Lamivudine (3TC), Stavudine (d4T), Didanosine (ddI), Zalcitabine (ddC), Abacavir (ABC), Emtrivine (FTC), Tenofovir (TDF), Delaviradine (DLV), Efavirenz (EFV), Nevirapine (NVP), Fuzeon (T-20), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir, Combivir  
30 (ZDV/3TC), Kaletra (RTV/LPV), Trizivir (ZDV/3TC/ABC), SCH-C, SCH-D, PRO

140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857, monoclonal antibodies, NB-2, NB-64, T-649, T-1249, and functional analog thereof.

7. The pharmaceutical composition of claim 4, wherein the compound is administered orally, rectally, nasally, topically, vaginally or parenterally.

8. The pharmaceutical composition of claim 1, wherein the G1 phase arresting agent is RAPA or HU.

9. A pharmaceutical composition for reducing effects of Human Immunodeficiency Virus (HIV) infection by decreasing expression of CCR5 surface receptors on mononuclear cells, the composition comprising a therapeutically effective amount of Rapamycin.

10. The pharmaceutical composition of claim 9, wherein the therapeutically effective amount of Rapamycin is from about 5mg/kg to about 50 mg/kg per day.

11. A method for inducing increased levels of anti-HIV  $\beta$ -chemokines in activated lymphocytes, the method comprising:

administering a composition comprising at least one G1 phase arresting agent in an effective amount to decrease expression of CCR5 surface receptors, wherein the decreased levels of CCR5 surface receptors reduces viral entry of HIV.

12. The method of claim 11, wherein the G1 phase arresting agent is a member selected from the group consisting of: sodium butyrate, aphidicolin, hydroxyurea (HU), olomoucine, roscovitine, tocopherols, tocotrienols, and rapamycin (RAPA).

13. The method of claim 11, wherein the G1 phase arresting agent is RAPA.

14. The method of claim 11, further comprising at least one HIV antiviral agent.

15. The method of claim 14, wherein the HIV antiviral agent is a nucleoside RT inhibitor, CCR5 inhibitors/antagonist, viral entry inhibitor or functional equivalent thereof.

5 16. The method of claim 13, wherein the at least one antiviral agent is a member selected from the group consisting of: Zidovudine (ZDV, AZT), Lamivudine (3TC), Stavudine (d4T), Didanosine (ddl), Zalcitabine (ddC), Abacavir (ABC), Emirivine (FTC), Tenofovir (TDF), Delaviradine (DLV), Efavirenz (EFV), Nevirapine (NVP), Fuzeon (T-20), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Nelfinavir  
10 (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir, Combivir (ZDV/3TC), Kaletra (RTV/LPV), Trizivir (ZDV/3TC/ABC), SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857, monoclonal antibodies, NB-2, NB-64, T-649, T-1249, and functional analog thereof.

15 17. The method of claim 13, wherein the compound is administered orally, rectally, nasally, topically, vaginally or parenterally.

18. A therapeutically effective method of combating a virus infection that is dependent of levels of CCR5 surface receptors, the method comprising:  
20 administering to a subject a therapeutically effective amount of a composition comprising a G1 phase arresting compound to inhibit CCR5 expression thereby reducing CCR5 surface receptors and replication of the virus infection.

19. The method of claim 18, wherein G1 phase arresting compound is a member  
25 selected from the group consisting of: sodium butyrate, aphidicolin, hydroxyurea (HU), olomoucine, roscovitine, tocopherols, tocotrienols, and rapamycin (RAPA).

20. The method of claim 18, further comprising administering an effective amount of at least one HIV antiviral agent.

30

21. The method of claim 20, wherein the antiviral agent is a nucleoside RT inhibitor, CCR5 inhibitors/antagonist, viral entry inhibitor or functional equivalent thereof.
- 5 22. The method of claim 21, wherein the antiviral agent is at least one member selected from the group consisting of: Zidovudine (ZDV, AZT), Lamivudine (3TC), Stavudine (d4T), Didanosine (ddl), Zalcitabine (ddC), Abacavir (ABC), Emirivine (FTC), Tenofovir (TDF), Delaviradine (DLV), Efavirenz (EFV), Nevirapine (NVP), Fuzeon (T-20), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Nelfinavir  
10 (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir, Combivir (ZDV/3TC), Kaletra (RTV/LPV), Trizivir (ZDV/3TC/ABC), SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857, monoclonal antibodies, NB-2, NB-64, T-649, T-1249, and functional analog thereof.
- 15 23. The method of claim 18, further comprising administering an effective amount of an HIV vaccine.
24. The method of claim 23, wherein the HIV vaccine and the G1 phase arresting agent are administered concurrently.
- 20 25. A method of maintaining viral control of an HIV infection, the method comprising:  
administering at least one G1 phase arresting compound in effective amount to reduce express of CCR5 surface receptors in an amount to reduce binding sites for  
25 HIV gp120.
26. The method of claim 25, wherein the G1 phase arresting compound is a member selected from the group consisting of: sodium butyrate, aphidicolin, hydroxyurea (HU), olomoucine, roscovitine, tocopherols, tocotrienols, and rapamycin  
30 (RAPA).

27. The method of claim 25, wherein the G1 phase arresting agent is rapamycin.
28. A pharmaceutical composition comprising a G1 phase arresting agent and a CCR5 antagonist compound, wherein the G1 phase arresting agent is rapamycin or  
5 hydroxyurea.
29. The pharmaceutical composition of claim 28, wherein the CCR5 antagonist compound is TAK-779.
- 10 30. The pharmaceutical composition of claim 29, wherein the amount of rapamycin or hydroxyurea is sufficient to synergically enhance the activity of TAK-779.
31. A method to synergically enhance the activity of a CCR5 antagonist  
15 compound, the method comprising administering the CCR5 antagonist compound in combination with a G1 phase arresting agent.
32. The method of claim 31, wherein the CCR5 antagonist compound is TAK-779 and the G1 phase arresting agent is rapamycin or hydroxyurea.

20

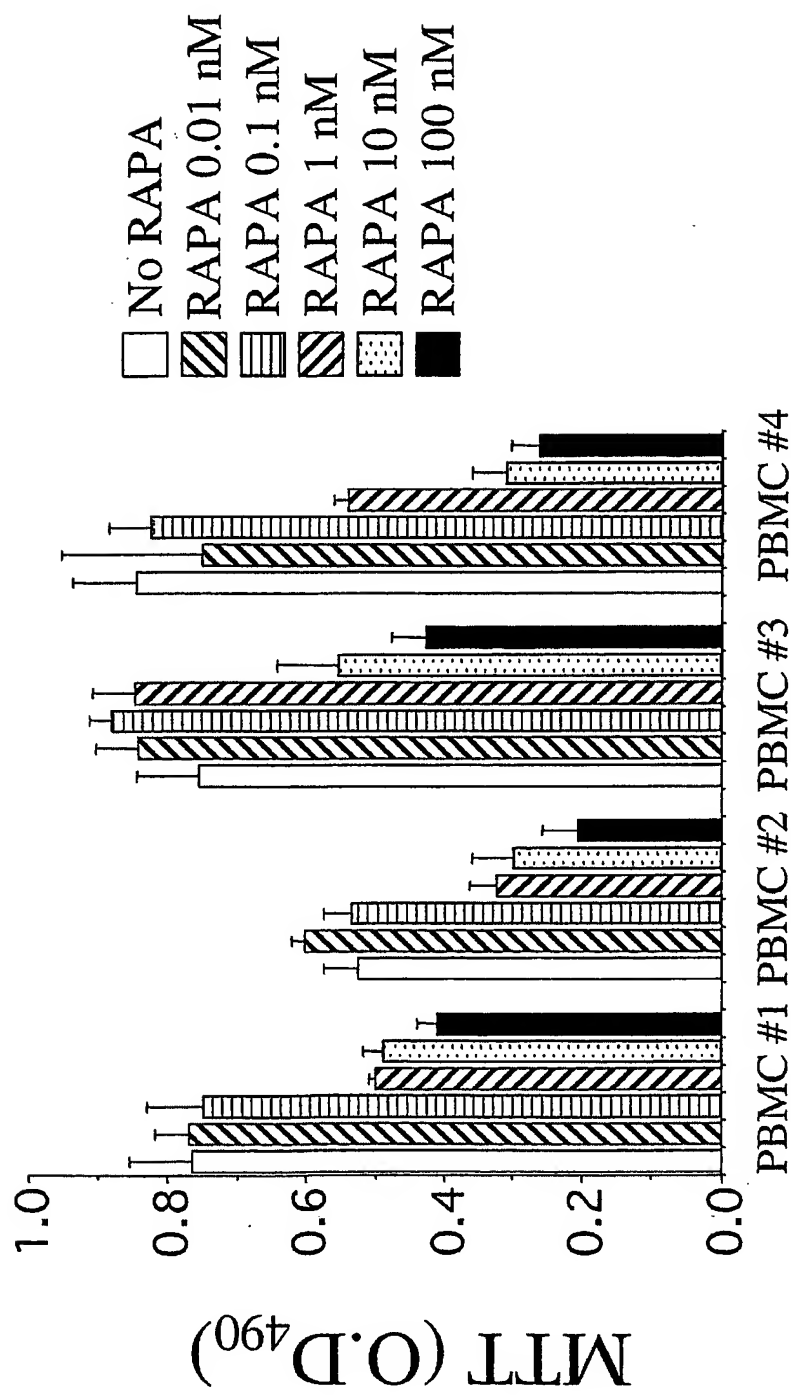


Figure 1

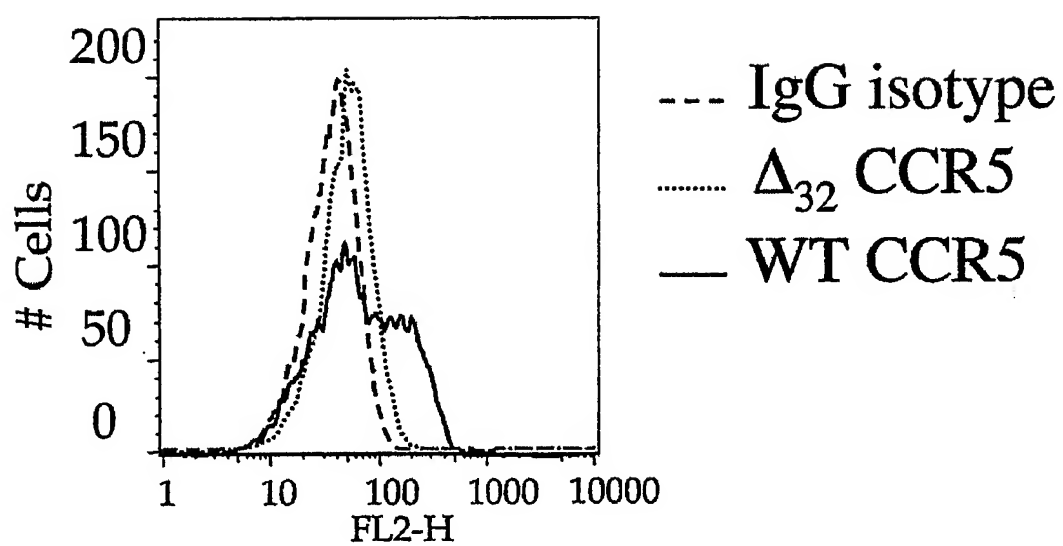


Figure 2A

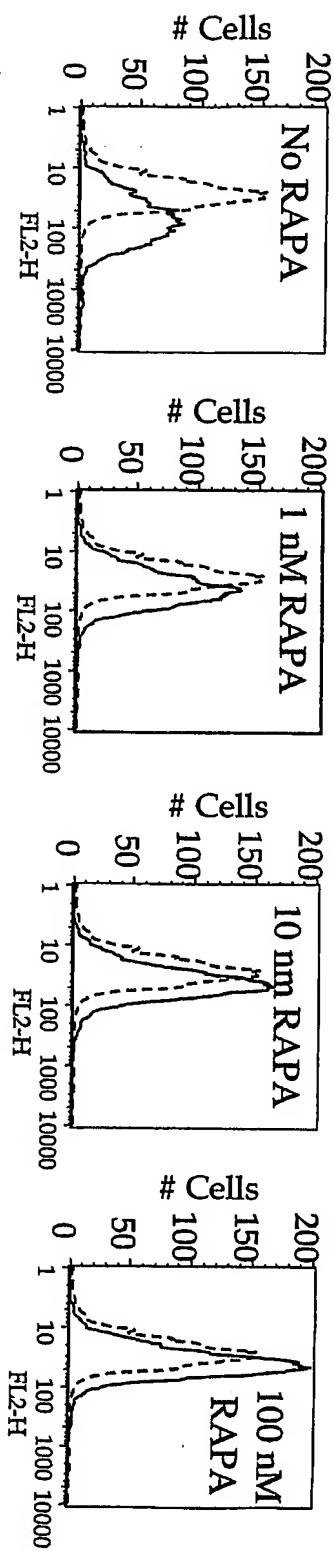


Figure 2B

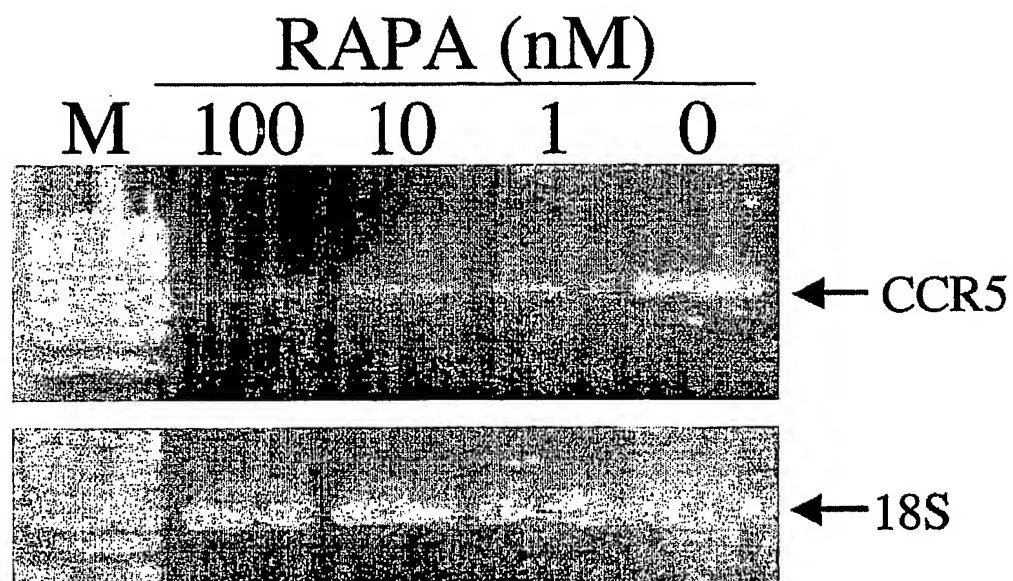


Figure 2C

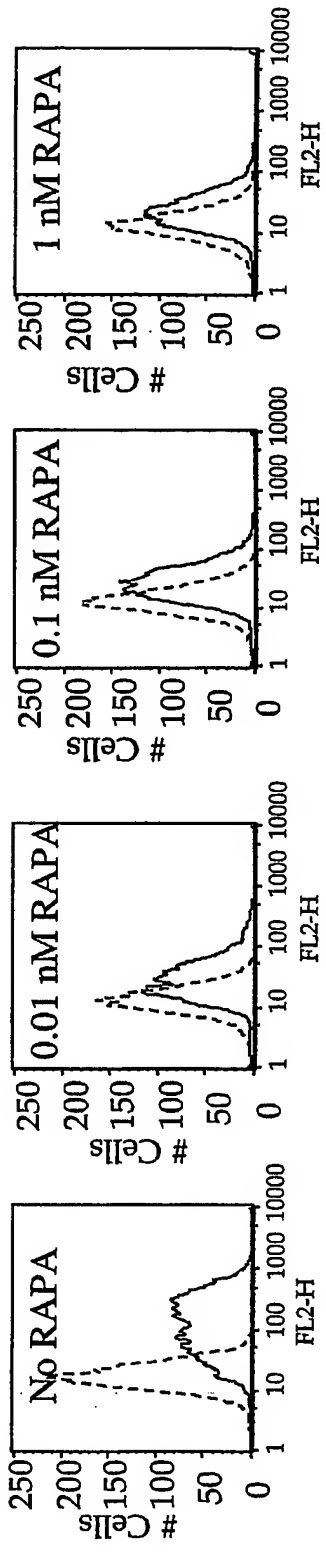


Figure 2D

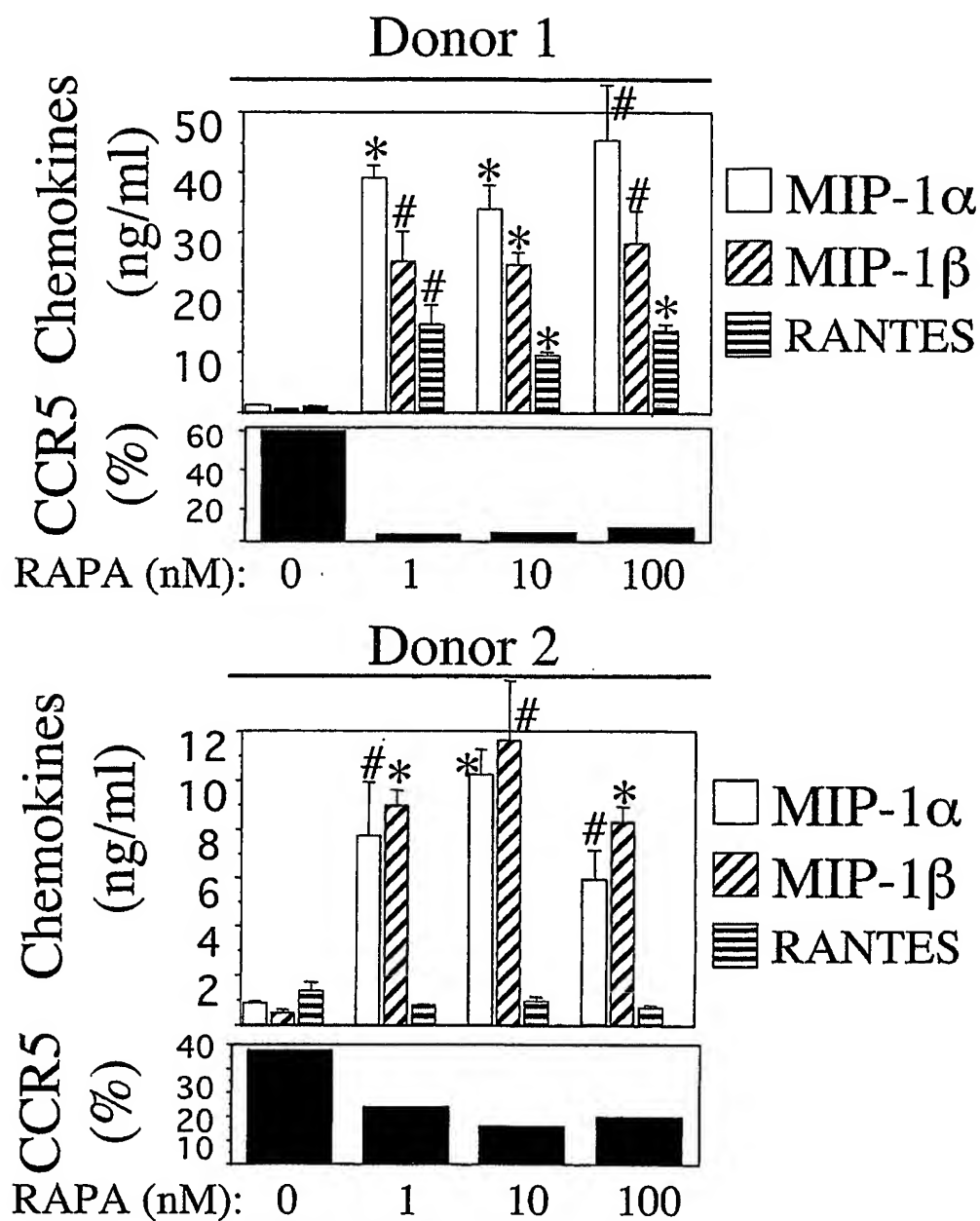


Figure 3A

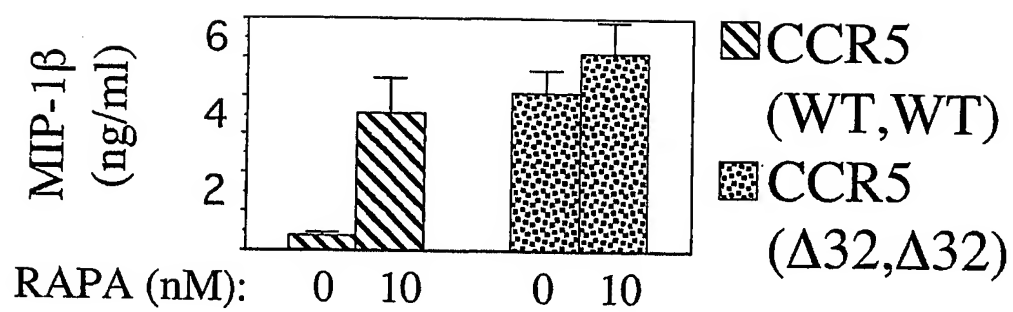


Figure 3B

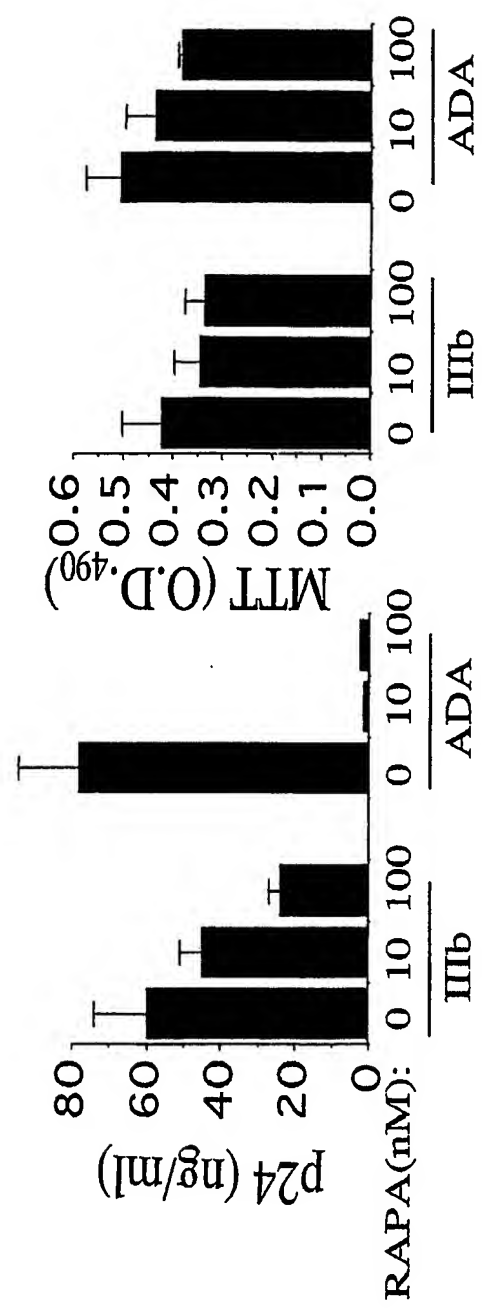


Figure 4A

IIIb		ADA	
NC	NC	+	+
+	-	-	-

Figure 4B

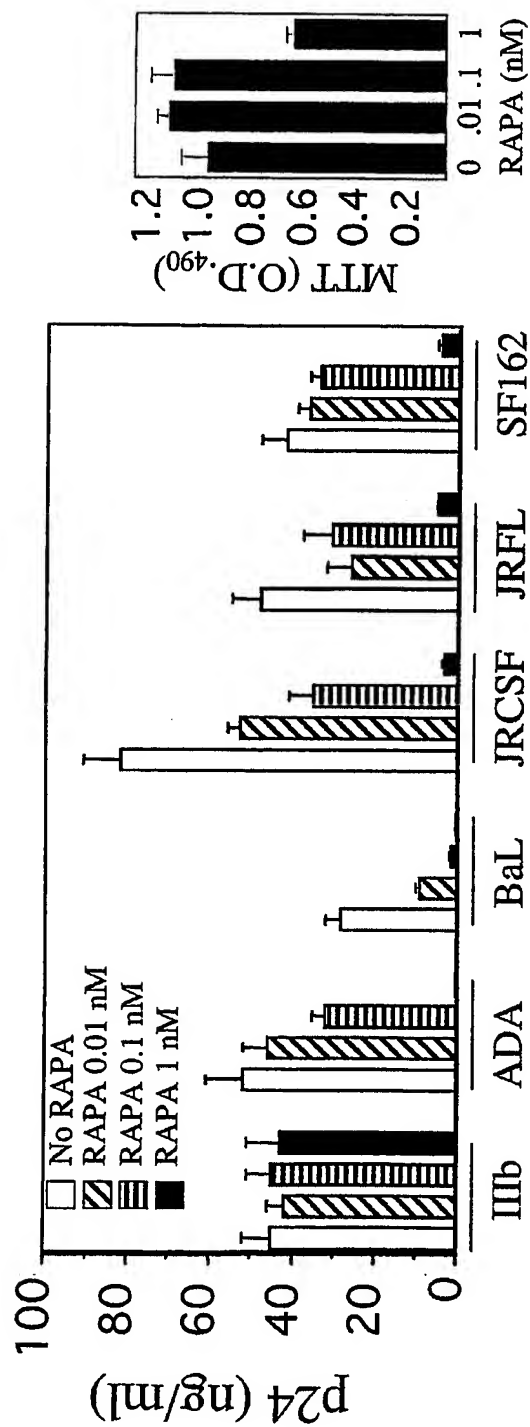


Figure 4C

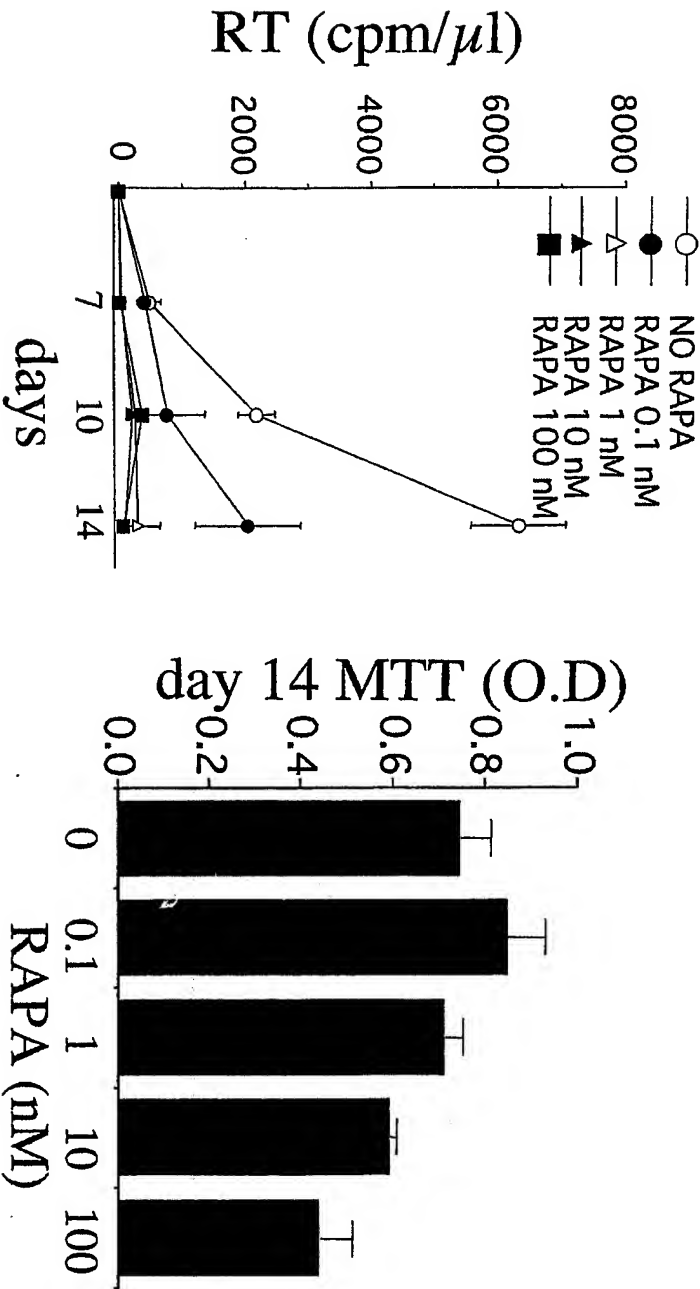


Figure 5

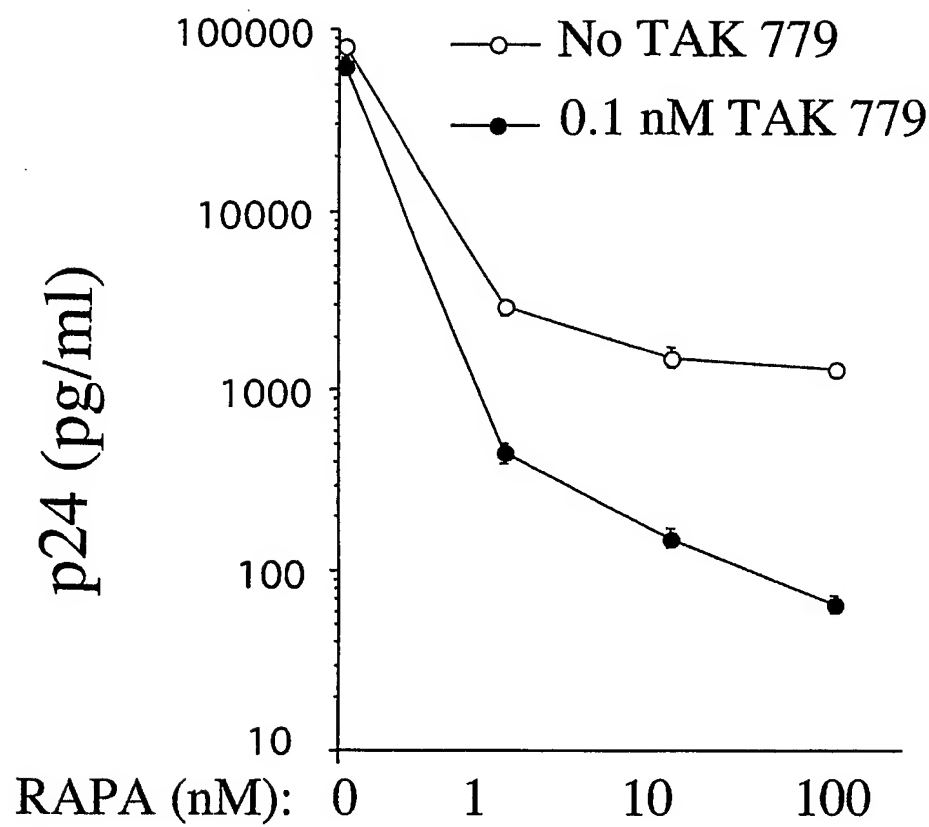


Figure 6

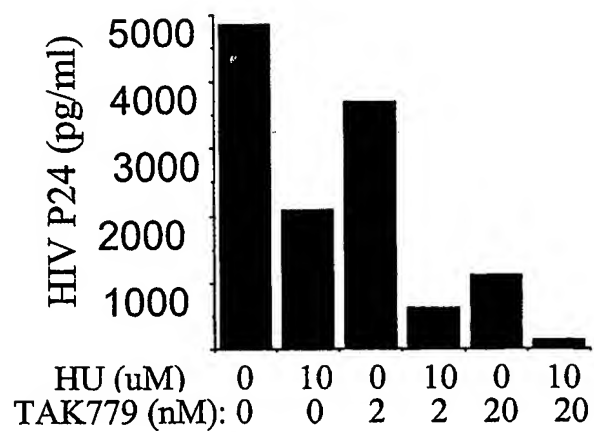


Figure 7

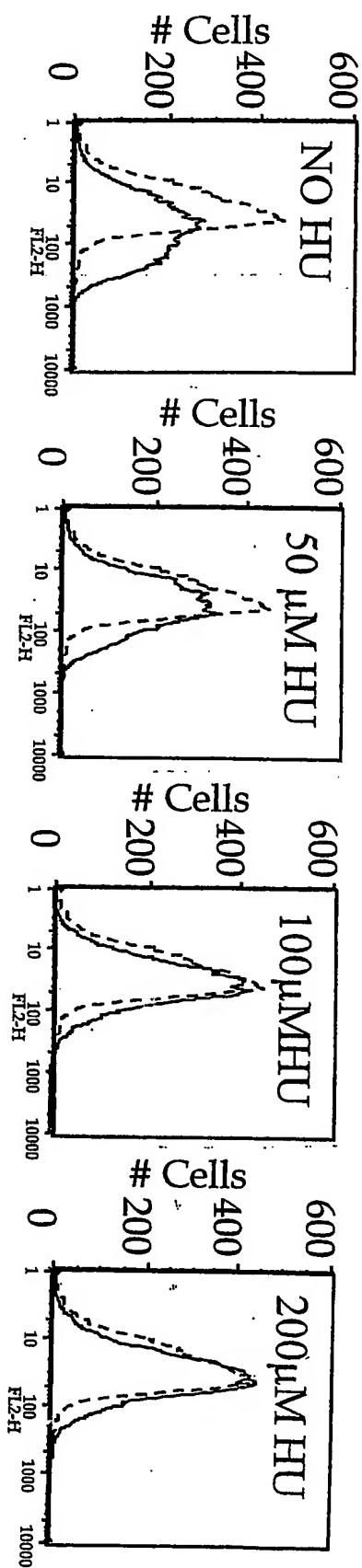


Figure 8A

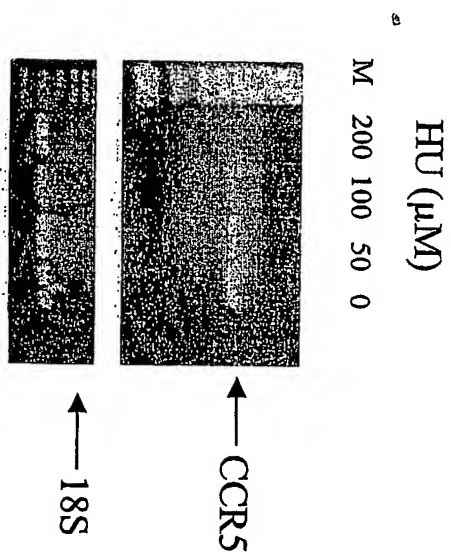


Figure 8B

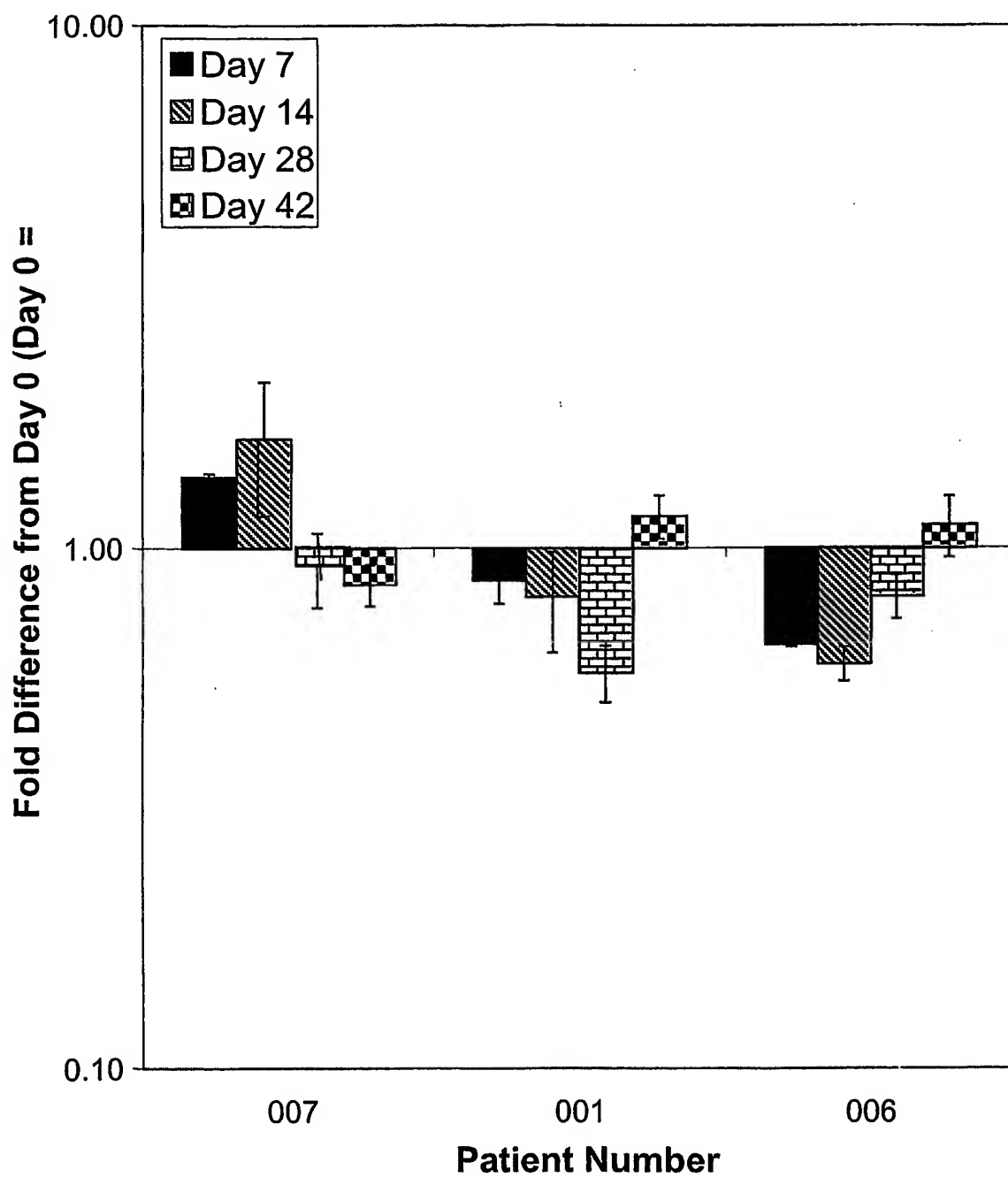


Figure 9

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
6 January 2005 (06.01.2005)

PCT

(10) International Publication Number  
**WO 2005/001027 A3**

(51) International Patent Classification<sup>7</sup>: **A61K 31/355**,  
31/35, 31/33, 31/435, 31/17, 31/385, 31/70

(21) International Application Number:  
PCT/US2004/015681

(22) International Filing Date: 17 May 2004 (17.05.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/471,453 16 May 2003 (16.05.2003) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

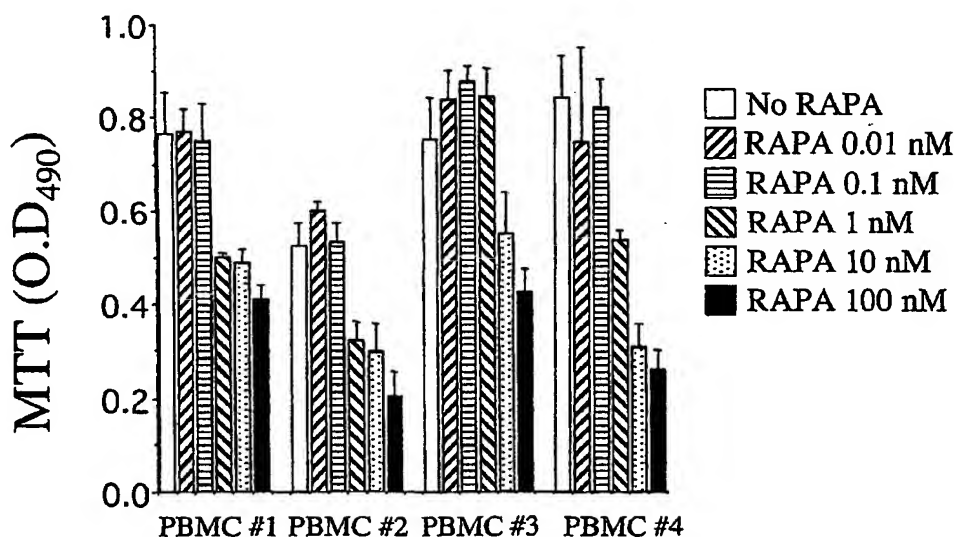
Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(88) Date of publication of the international search report:  
26 January 2006

[Continued on next page]

(54) Title: COMPOSITIONS FOR DOWN-REGULATION OF CCR5 EXPRESSION AND METHODS OF USE THEREFOR



(57) Abstract: The present invention relates to the downregulation of surface receptor CCR5 expression through manipulation of the cell cycle in activated lymphocytes by administering a composition that arrests the G1 phase of the cell cycle, thereby reducing receptor sites for entry of HIV into T cells, and thus, the effects of HIV. Further, a composition is disclosed the includes a G1 phase arresting agent and an antiviral agent, wherein the combination synergically enhances the activity of the antiviral agent.

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## INTERNATIONAL SEARCH REPORT

Intern \_

PCT/US04/15681

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/355,35,31/33,31/435,31/17,31/385,31/70

US CL : 514/459,458,183,277,588,45,441

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/459,458,183,277,588,45,441

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CAPLUS, MEDLINE, BIOSIS, EMBASE, search terms: hydroxyurea, rapamycin, CCR5, TAK-779

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 99/48504 (LISZIEWICZ et al) 30 SEPTEMBER 1999 (30.09.1999). the entire document, particularly, the abstract, the examples at pages 11-12 and the claims.	1-8, 11-22, 25-28 9,10,23,24,29-32
X -- Y	WO 99/47146 (LISZIEWICZ et al), 23 SEPTEMBER 1999 (23.09.1999). the entire document, particularly, the abstract, the claims, and the examples.	1-8, 11-22,25-28 9,10,23,24,29-32
X -- Y	WO 94/05300 (VEZINA et al), 17 MARCH 1994 (17.03.1994). the entire document, particularly, the abstract, pages 7-17, and the claims.	1-22,25-28 23, 24,29-32
Y	US 6,096,780 A (SHIRAISHI et al.), 01 AUGUST 2000 (01.08.2000). the entire document, particularly, the abstract, columns 1-2, and the claims.	1-32



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
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Date of the actual completion of the international search

07 November 2005 (07.11.2005)

Date of mailing of the international search report

02 DEC 2005

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